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Hyper-osmolarity and calcium chelation: Effects on cystic fibrosis mucus

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Chemical compounds studied in this article:
Carbachol (2-carbamoyloxyethyl(trimethyl) azanium
chloride) (PubChem CID: 5831)
d-mannitol (PubChem CID:6251)
Erdosteine (PubChem CID: 65632)
2,2′,2″,2‴-(Ethane-1,2-diyldinitrilo)tetra-acetic acid (PubChem CID: 6049)
N-acetyl-l-cysteine (PubChem CID: 12035)
Sodium bicarbonate (PubChem CID: 767)
Prostaglandin E_2 (PubChem CID: 5280360)

ABSTRACT

A non-functional Cystic Fibrosis Transmembrane conductance Regulator (CFTR) leads to the disease cystic fibrosis (CF). Although the CFTR is expressed in multiple organs, pulmonary disease is the major cause of illness and death in patients with CF. Stagnant mucus, causing airway obstruction, bacterial overgrowth, persistent inflammation and tissue destruction characterizes the disease, but how the defect in CFTR function is coupled to the mucus phenotype is still controversial. We have recently shown that bicarbonate ions passing through CFTR are necessary for proper unfolding of the MUC2 mucin, thus highlighting the importance of bicarbonate ion transport via the CFTR and the ability of these ions to raise the pH and chelate calcium bound to the mucin as the important steps in forming normal mucus. In order to find potential CF treatments and expand our knowledge about the usefulness of bicarbonate as an active ingredient in formulations to alleviate mucus plugging, we used an Ussing-type chamber and explants from the F508del-CFTR mutant mouse ileum to test the effect of calcium chelators on mucus attachment, either in isolation or in combination with osmolytes such as mannitol or hypertonic saline. We found that increasing the concentration of bicarbonate, both alone or in combination with increased osmolarity of the solution, detached the otherwise attached CF mucus.

1. Introduction

Cystic fibrosis (CF) is caused by mutations in the ion channel Cystic Fibrosis Transmembrane conductance Regulator (CFTR) (Riordan et al., 1989), progressive bronchiectasis and lung damage being the main causes of morbidity and mortality (Chmiel and Davis, 2003). The disease causing mutations affect permeability to chloride and bicarbonate (Quinton, 2001) and lack of bicarbonate causes formation of attached mucus (Garcia et al., 2009; Gustafsson et al., 2012a). In CF the attached, stagnant mucus leads to bacterial overgrowth, infection and inflammation. The inflammatory response can increase the number of mucus secreting cells, stimulate mucus secretion and further influence mucus properties (Rogers, 1994). Many CF patients also suffer from distal intestinal obstruction syndrome (DIOS), where attached mucus obstructs the distal ileum and leads to bacterial overgrowth. Consequently, effective treatments should be aimed at preventing or reversing the attachment and stagnation of mucus.

We have shown that a MUC2 N-terminal recombinant protein consisting of the VWD1-D2-D′D3 domains formed large aggregates at pH 6.2 in the presence of Ca^{2+}. The aggregates were dissolved upon Ca^{2+} chelation and pH increase. Chelation of Ca^{2+} was achieved by 2,2′,2″,2‴-(Ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA) or bicarbonate (Ambort et al., 2012), which lead us to test Ca^{2+} chelation in the F508del-CFTR mutant mouse ileum (van Doorninck et al., 1995). We could show that bicarbonate ions passed through CFTR are necessary to establish proper unfolding

Abbreviations: Ab-PAS, Alcian blue-Periodic Acid Schiff; CF, cystic fibrosis; CFTR, Cystic Fibrosis Transmembrane conductance Regulator; COPD, chronic obstructive pulmonary disease; DIOS, distal intestinal obstruction syndrome; EDTA, 2,2′,2″,2‴-(Ethane-1,2-diyldinitrilo)tetraacetic acid; F508del-CFTR, Fenylalanin 508 deletion in CFTR; MUC2/MUC5AC, mucin 2/mucin 5AC; NAC, N-acetyl-L-cysteine; PGE2, prostaglandin E2

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of the Muc2 mucin, as newly formed mucus became attached in the WT mouse ileum when the basolateral buffer was depleted of bicarbonate ions. When mucus was secreted into apical isotonic 115 mM bicarbonate buffer the CF ileal mucus became easily aspirated and penetrable to beads the size of bacteria (Gustafsson et al., 2012a). Furthermore, forming mucus in an apical buffer with 20 mM EDTA normalized the attached CF mucus into a more easily aspirated phenotype (Gustafsson et al., 2012a). To find potential CF treatments, we tested calcium chelators, either in isolation or in combination with mannitol, since clinical trials on CF patients have shown that inhaled dry powder mannitol improves mucociliary clearance (Aitken et al., 2012; Robinson et al., 1999).

N-acetyl-L-cysteine (NAC) is used as a mucolytic drug in respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and CF (Rogers, 2007) and erdosteine is an expectorant available for treatment of chronic bronchitis and COPD (Cazzola et al., 2010). Since they are defined as mucolytics, we tested the ability of these substances to detach F508del-CFTR mutant mouse ileal mucus.

The treatments deemed promising are the ones which (1) make the F508del-CFTR mutant mouse ileal mucus as easily aspirated as in the WT mouse ileum, (2) are also effective on preformed mucus and (3) do not cause expansion of the mucus, since expansion may cause airway plugging. Our results indicate that calcium chelation by bicarbonate or EDTA in combination with hypertonic buffer, either achieved by NaCl or mannitol causes effective detachment while mucus expansion is kept at a minimum. NAC and erdosteine had no effect on mucus attachment.

2. Materials and methods

2.1. Animals

Male and female (age 8–16 weeks) homozygous F508del-CFTR mutant mice on C57BL/6 background were bred as heterozygotes at the University of Gothenburg. Mice were housed in individually ventilated cages under controlled temperature (21–22 °C), humidity and 12-h light/dark cycle under specific pathogen-free conditions, maintained as described (van Doorninck et al., 1995) and given regular water 2–3 days before the experiments. All mice were killed by cervical dislocation under isoflurane anesthesia. Ethical approval was granted by the Laboratory Animal Ethics Committee, University of Gothenburg, and experimental animal care was in accordance with their guidelines.

2.2. Explants

Intestinal explants were prepared and mounted as described previously (Gustafsson et al., 2012b). Briefly, explants were mounted between two chambers, Krebs–glucose buffer was perfused basolaterally and Krebs–mannitol was added apically. Activated charcoal particles in Krebs–mannitol buffer were added and allowed to sediment on top of the mucus to visualize the mucus surface through a stereomicroscope at 40× magnification (Leica MZ125, Wetzlar, Germany). For full removal, mucus was aspirated with a Pipetman P200 (Gilson, Middleton, WI, USA) set to 150 μl and using a 20–200 μl tip. Tissue viability was monitored by measuring PD using electrodes (Ref201; Radiometer, Copenhagen, Denmark) connected to the chamber by agar bridges (4% agar, 0.9% NaCl).

2.3. Mucus thickness measurements

Mucus thickness was measured as previously described, from the mucus surface to the villi tips every 20 min for 60 min (Ermund et al., 2015, 2013; Gustafsson et al., 2012a,b). All apical incubations were done for 60 min. The mucus already formed when tissue was mounted is denoted preformed mucus. In the figures, the mucus thickness at 60 min is illustrated by white bars and denoted “Pre”. To evaluate mucus properties, the whole apical volume was aspirated using a plastic Pasteur pipette (PP-101, outer tip diameter 0.9 mm, inner tip diameter 0.7 mm, maximum volume 800 μl; Cellprojects, Sutton Valence, UK). The pipette tip was placed at the edge of the circular opening without touching the mounted tissue and kept in place while the bulb was released for approximately 3 s, thus extracting all removable liquid. The remaining mucus thickness, presented by black bars and denoted “Post” in the figures, was measured after refilling the apical chamber with 150 μl Krebs–mannitol to ensure that any remaining mucus material did not collapse and adding new charcoal particles. Finally, all mucus was removed and the villus height was measured from the epithelium between the villi to the villi tips. Total mucus thickness is presented as the sum of villus height and mucus on top of the villi.

In experiments where effects on mucus secreted into buffer containing treatments were evaluated, mucus was measured at 0 min, preformed mucus was removed and villus height measured before mucus secretion was induced by basolateral perfusion with 10 μM carbachol and 10 μM PGE_2 in Krebs–glucose at 20 min (blue arrow in figures) for 40 min. Mucus properties were evaluated as for preformed mucus. All apical incubations were done for 60 min.

2.4. Preparation of apical buffers

Calcium was omitted from buffers containing EDTA and osmolarity was adjusted by exchanging the corresponding molar concentration of sodium chloride for bicarbonate in the buffers with increased bicarbonate concentration. In the hyper-osmolar bicarbonate buffers, bicarbonate was added to the standard Krebs–mannitol buffer. Buffers with increased concentration of bicarbonate were first bubbled with carbogen gas (5% CO_2, 95% O_2) and then the pH was adjusted to 7.4. Some of the bicarbonate will evaporate as CO_2 gas, but traditionally, concentrations are given as original bicarbonate concentration. For further information about bicarbonate buffers, see Gustafsson et al. (2012a).

2.5. Histological analysis

Tissue histology and mucus depletion from crypt goblet cells were evaluated in explants fixed in methanol-Carnoy after completed experiments. Paraffin-embedded tissue was cut in 4 μm sections and Alcian blue-Periodic Acid Schiff (Ab-PAS) stained. Images were acquired using an Eclipse E1000 microscope with a Plan-Fluor 40×/0.75 DIC objective (Nikon, Amstelveen, The Netherlands).

2.6. Reagents

Erdosteine (2-[2-Oxo-2-[(2-oxothiolan-3-yl)amino]ethyl]sulfanylacetic acid) was acquired from Abcam (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO).

2.7. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.) for n animals. The Mann–Whitney test was used to test differences between two groups. When the number of animals in the compared groups was too small ($n=3$) for a non-parametric test, the Student's t-test was used. Statistical significance was accepted when $P<0.05$.